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14. ABSTRACT

MIC-1/GDF-15 is a newly identified member of the TGF-beta superfamily of growth and differentiation factors. MIC-1/GDF-15 is highly expressed in both animal models and patients with breast cancer. MIC-1/GDF-15 is induced by chemotherapy. In order to understand the role of MIC-1/GDF-15 in breast cancer development the grant sought to breed wild-type and GDF-15 null mice into two mice lines with a genetic predisposition for breast cancer formation the (TgN(C3-1-TAg) and APC-min mouse lines. The project has had some setbacks but has begun the breeding to obtain the animals of interest to examine the effect of GDF-15 loss on tumor initiation, progression and metastasis.

15. SUBJECT TERMS
MIC-1, GDF-15, Breast Cancer

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Introduction:

Human MIC-1 and its murine ortholog GDF-15, are divergent members of the TGF-ß superfamily, a large family of secreted molecules required for normal development, differentiation and tissue homeostasis from flies to humans (*1*). MIC-1 and GDF-15 were each independently identified by a number of groups and have been reported also as hPDF (*2*), hPLAB (*3*), hPTGF-ßPL (*4*), and hNAG-1 (*5*). The normal expression patterns of *MIC1* and *Gdf15* are similar but not identical, with strong expression of *MIC1* in placenta and prostate, and less in liver, kidney and brain, while *Gdf15* is expressed in normal mouse liver and kidney (*1*), as well as in rodent choroid plexus, placenta, prostate, intestinal epithelium, bronchi and bronchioles, and vascular endothelium(*6, 7*). Activities ascribed to GDF-15/MIC-1 include inhibiting LPS-induced TNF production from macrophages (*8*), promoting survival of midbrain dopaminergic and raphe serotonergic neurons in vitro and in vivo (*9*), preventing cell death of cerebellar granule neurons (*10*), inducing chondrogenesis and early endochondral bone formation in subcutaneous implants (*2*), and inhibiting the proliferation of primitive hematopoietic progenitors (*3*). Since *Gdf15*$^{-/-}$ mice are apparently normal, fertile and exhibit no developmental abnormalities or defects in liver regeneration or repair, however the normal, non-redundant functions of GDF-15 are still unclear (*1*).

GDF-15/MIC-1, like all members of the TGF-ß superfamily, is synthesized as a preproprotein (*8*). Following disulfide-linked dimerization in the endoplasmic reticulum, the dimeric precursor is cleaved by proteases at a conserved tetra basic proteolytic processing site at amino acid 196. Cleavage results in release of the propeptide from the C-terminal biologically active portion of the molecule. Unlike other TGF-ß family members, however, the pro-peptide of GDF-15/MIC-1 is not required for proper folding and secretion (*11*). The most highly related, canonical members of the TGF-ß superfamily, including the TGF-ßs themselves, the activins, the bone morphogenetic proteins, and certain of the growth/differentiation factors signal through heteromeric complexes of type I and II serine/threonine kinase receptors (*12*). Activation of these receptors results in phosphorylation and activation of second messenger transcription factors known as Smads as well as other signaling pathways(*13*). The most divergent members of the TGF-ß superfamily, the glial cell line-derived neurotrophic factor (GDNF) subfamily, however, signal through the GDNF family receptors alpha and the Ret receptor tyrosine kinase, subsequently activating only non-Smad pathways (*14*). The identities of the actual GDF-15/MIC-1 receptors are believed to be TGF-ß receptors; several reports document activation of TGF-ß responsive promoters and growth inhibition by recombinant or transfected GDF-15/MIC-1 in cell lines with intact TGF-ß signaling pathways, but not in certain cells lacking TGF-ß receptors type I, II or Smad4 (*15*).

The founding member of the TGF- ß superfamily, TGF-ß1 itself, is a potent growth inhibitor, initiating apoptosis and suppressing tumorigenesis in many cell types. Inactivating mutations in the TGF-ß

receptors or its downstream signaling partners such as Smad4 have been described in a number of gastrointestinal cancers. Such mutations render the cancer cells refractory to growth inhibition, and also result in over-expression of TGF-ß by the tumor cells. The consequence of this is immunosuppression, increased angiogenesis in the tumor, enhanced invasiveness, and metastasis (39) (40-43). Although GDF-15/MIC-1 is highly divergent from TGF-ß at the primary amino acid level, it shares many TGF-ß-like characteristics, from immunosuppression (8), to over-expression in cancer cells (24, 25), to growth inhibition (14) and promotion of invasiveness (28). And indeed, some investigators have concluded that GDF-15/MIC-1 signals through the TGF-ß receptors (14). Moreover, GDF-15/MIC-1 expression is highly associated with expression of the stress sensing, growth-suppressing transcription factor p53. Taken together, these data are highly suggestive that GDF-15/MIC-1 may play a role in regulating normal or cancerous cell proliferation, although the evidence is far from definitive. Many of the growth suppression studies have been performed by transfection or infection of cells to produce autocrine production of MIC-1/GDF-15, while some have used only partially purified protein fractions. To date there is no evidence that MIC-1/GDF-15 activates the Smad2/3/4 pathway used by the TGF-ß receptors. Neither has there been an effort to determine the role of MIC-1/GDF-15 in cancer initiation, progression and metastasis using transgenic or knockout approaches. Thus both the true identity of the MIC-1/GDF-15 receptors and the actual function of MIC-1/GDF-15 in both homeostasis and carcinogenesis are still unclear.

In addition to the previously mentioned trophic functions on neurons and growth inhibition of cell lines, several lines of evidence suggest GDF-15/MIC-1 may regulate proliferation and apoptosis in normal, injured and transformed cells *in vivo*. Firstly, GDF-15 is constitutively expressed in a variety of tissues in uninjured animals, and notably in cells undergoing apoptosis in normal murine and human intestinal villi (7). Secondly, GDF-15/MIC-1 is potently induced by numerous stressors in a wide variety of tissues (*16*), the stress-sensor/growth inhibitor p53 (*17*) (*18*) and by apoptosis-promoting, anti-tumorigenic substances such as etoposide (*15*), resveratrol (*19*), certain non-steroidal anti-inflammatory drugs (NSAIDs) (7) (*20*), genistein (*21*), troglitazone (*22*), diallyl disulfide (*23*), 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN) (*24*), and 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F-203) (*25*). Thirdly, increased MIC-1 expression and serum MIC-1 levels were positively correlated with human colorectal cancer tumor-stage-metastasis severity according two studies (*26*) (*27*), although expression was reduced in tumors as compared to normal intestinal epithelium in another (7). Similarly, MIC-1 was found to be over expressed in prostate cancer, although decreased expression was observed in higher grade cancer (*28*). Finally, over expression of GDF-15/MIC-1 by transfection or infection promoted apoptosis in human breast (*29*), prostate (*30, 31*) (*32*) and colorectal (7) cell lines, increased invasiveness of gastric cancer cell lines (*33*), and abolished tumorigenicity of a glioblastoma cell line in nude mice (*18*). Such functions are similar to and overlap those observed by over-

expression of constitutively active TGF-β receptors(*13*). Table 1 summarizes the current literature regarding the role of GDF-15 in cancer.

Table 1:Reported roles of MIC-1/GDF-15 in Cancer

Organ site of tumor development	**Reported MIC-1/GDF-15 actions/ role**	**Reference**
Brain	Glioblastoma produce and secrete GDF-15/ MIC-1 with anoxia. Induced in brain lesions.	(*18*) (*10*)
	GDF-15/ MIC-1 has anti-apoptotic effects on cerebellar granular cells partly through AKT activation	(*9*) (*10*)
	GDF-15/ MIC-1 is an in vivo growth factor for dopaminergic neurons	(*9, 10*)
Breast	MIC-1 is induced in vivo by AKT activation in breast cancer cells	(*34*)
	MIC-1 is induced immediately following neo-adjuvant chemotherapy	(*35*)
Colon	MIC-1 predicts outcome of colon cancer and degree of expression correlates with tumor stage and burden	(*27*)
	MIC-1 expressed in colon cancer (gene array study)	(*26*)
	p53 activation results in MIC-1 tumor expression *in vitro* and *in vivo*	(*36*)
	Multiple chemotherapeutic agents induce MIC-1 in colon cancer cell lines	(*7, 15*)(17-26)
	NSAIDs induce MIC-1 in colon. Implicate MIC-1 in NSAID anti-polyp function	(*7*)
Gastric Cancer	Increases invasiveness of gastric cancer	(*33*)
	Increased MIC-1 expression in NSAID-induced gastric cancer apoptosis	(*37*)
Kidney	MIC-1 induced following multiple acute injuries and carcinogen exposures	(*16*)
Liver	MIC-1 induced following multiple acute liver injuries and carcinogen exposures	(*1, 16, 38*)
Lung	MIC-1 expressed by multiple lung cancer cell lines	(*24*)
	Induced following lung injury, chemotherapy and carcinogen exposures	(*16*)
Ovary	MIC-1 is growth inhibitory for ovarian tissue and involved in NSAID-mediated growth inhibition	(*39*)
Oral Cavity	NSAID induce apoptosis through MIC-1 induction	(*40*)
Pancreas	Increased expression in pancreatic cancer	(*41*)
	Increased MIC-1 is a marker of periampullary malignancies	(*42*)
Thyroid	MIC-1 is expressed by follicular carcinomas	(*43*)
Prostate	Association between a non-synonymous change (H6D) in the MIC-1 gene and prostate cancer.	(*44*)
	MIC-1 induces prostate cancer cell apoptosis	(*30*)
	Increased MIC-1 serum levels associated with tumor progression and burden	(*31*) (*32*)
	MIC-1 is expressed in prostate cancer	(*45*)

C. Body

Overview: The project initially had the misfortune of having the colony of GDF-15 null animals we had generated for this study (approximately 60 GDF-15 null animals) dying while in the 60 day University imposed quarantine. We subsequently were able to re-obtain two breeding pairs from a collaborator. We have been working to generate sufficient quantities of the mice of interest and are well along in accomplishing this goal. As well, we have encountered some problems in breeding the APC-min mouse that have a markedly shortened longevity (less than 4-6 months due to the formation of cancers) and therefore have been slow in obtaining these animals (GDF-15 (-/-), APC-min (+/-)). We have generated some F1 offspring (GDF-15 (+/-), APC-min (+/-)) but these died before we were able to breed them (at 4 months of age). Overall, we plan to continue the proposed project utilizing other funds including a recent gift of approximately $100,000 to the PI from the Papanicolaou Corps an outstanding cancer research support group. We anticipate that a publication of the findings of this grant will be completed, however, it is clear that at least an additional 2-year period will be required to complete these studies.

Female C57BL/6MIC-1 -/- animals are in the process of being bred to two distinct commercially available (Jackson Labs, Bar Harbor, ME) genetically modified mice lines that demonstrate an increased incidence of breast malignancies. The first mouse line over-expresses SV40 large T antigen on a breast -specific promoter (TgN(C3-1-TAg). The second mouse line has a modified APC gene (adenomatous polyposis coli), i.e. the Min (multiple intestinal neoplasia) mouse. It is unusual for either of the breast tumor-prone mouse lines to survive far beyond 6 months of age due to the development of malignancies. Both commercially available mouse lines are in the process of being bred into a MIC-1 -/-background. F1 animals will be screened and bred again into the MIC-1 -/- mice to obtain MIC-1/GDF-15 null mice that also express large T antigen (TgN(C3-1-TAg), gdf15-/-) or also possess a modified APC gene (Min+/-, gdf15-/-). Age and sex-matched (TgN(C3-1-TAg), gdf15-/-), (Min+/-, gdf15-/-),(TgN(C3-1-Tag), gdf15+/+), and (Min +/-, gdf15+/+) mice will be obtained. Ten female animals of each category will be sacrificed and subjected to necropsy at 1, 3 or 5 months of age. A determination of whether the number and/or frequency of breast and other tumor formation occur in animals without MIC-1 will be determined.

We now have large numbers of gdf15(-/-) mice that are the product of the heterozygous expansion of the gdf15+/- matings (Fig. 1). We currently are genotyping these animals using a PCR protocol we have developed to the c-terminal domain of the gdf15 gene and to the G418 resistance insert (Fig. 2). As well, we are able to genotype both min (+/-) and Tag mice (Fig 3). Following this round of matings as well as gdf15 heterozygous matings to each of the above tumor-prone lines we should be close to generating the genotypes for the proposed study. Of note, we have encountered some difficulty in the viability of min heterozygote mice and will plan to also modify our approach and also examine the effect of gdf15 -/- on the DMBA/ medroxy-progesterone acetate breast cancer model. This

additional objective will allow examination of gdf15 in a tumor progression model in shorter order.
Additional note: the min and T-antigen lines were chosen for the proposed experiment as they get a number of malignancies in addition to breast cancer and it is our intention to fully analyze these animals. We have, for example, received additional unrestricted funding from regional cancer societies that will help complete this challenging but important study.

Current numbers (9/20/2005) of mice for proposed experiments:

1) **GDF-15 (-/-) x GDF-15 (-/-)**
(40 Breeding Pairs)

↓

Multiple offspring being used for experiments and colony expansion

2) **GDF-15 (-/-) x APC min (+/-)**
(16 Breeding Pairs (8 APC min male x 16 GDF-15 (-/-) females)

↓

-Multiple offspring awaiting weaning.
-two GDF-15 (+/-) APC min (+/-) died of cancer prior to reproducing

3) **Tag x GDF-15 (-/-)**
(4 Breeding pairs, Tag male cross 4 GDF-15 (-/-) females)

↓

Pregnant females awaiting offspring

4) **Tag GDF-15(+/-) x GDF-15 (-/-)**
(1 breeding pair, female [Tag, GDF-15 (+/-)] x GDF-15 (-/-) male)

Figure 1. Summary of completed breeding scheme.

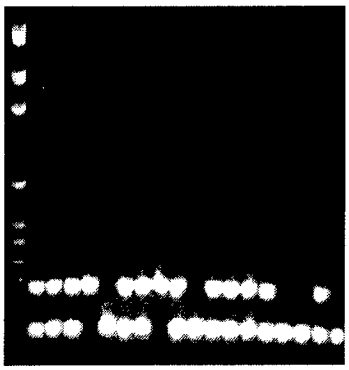

Figure 2. PCR-based genotyping of GDF-15 (-/-) animals.

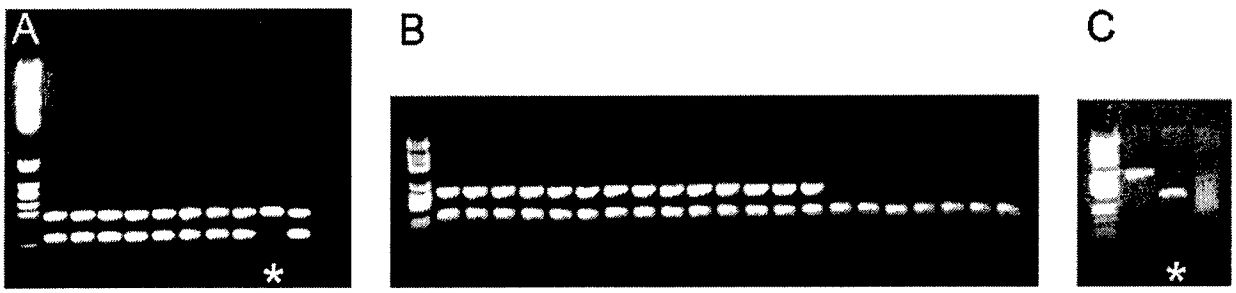

Figure 3. PCR-based genotyping demonstrating genotyping of all mouse lines proposed in the grant: A) GDF-15 heterozygous and null genotypes by PCR. 230 bp fragment corresponds with gdf-15 null allele, smaller fragment wild-type allele. Single *Gdf15* homozygous null among heterozygotes shown (asterix). B) PCR genotyping demonstrating two products in C3-1-TAg transgenic mice, the 474 bp product specific to the C3-1-TAg transgene, and the 200 bp Tcrd internal control (protocol adapated from a JAX protocol), while only the latter is amplified in non-transgenic mice. A fraction of the C3-1-TAg mice were GDF-15 (+/-) (data not shown. C) PCR genotyping demonstrating the 600 bp product amplified in mice wild-type for the Apc locus, and an additional 340 bp product in a mouse heterozygous for the Apc^{Min} mutation (protocol adapted from a JAX protocol). This $Apc^{Min/+}$ mouse was also heterozygous for the Gdf15 deletion (data not shown).

Key research accomplishments:

-Marked mouse colony expansion to generate the mice of interest

-Development of a pcr-based genotyping model for gdf15 animals. Below is a photo of a 2% agarose gel following PCR amplification of the c-terminal gdf15 region and the G418 cassete. Lanes with larger product only represent gdf15-/-.

Reportable outcomes:

Additional data required prior to publication.

Conclusions:

We have greatly expanded our colony following an unfortunate start and are on the way to generating the mice of interest proposed in the application.

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